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14. ABSTRACT

One of the most significant barriers to establishing an early ovarian cancer (OVCA) detection test is the difficulty of identifying patients with early stage OVCA. In earlier approaches to develop an early detection test for OVCA using serum levels of CA-125, traditional transvaginal ultrasound and their combination did not improve the detection of OVCA remarkably. The limitation of CA-125 is that it is non-specific in detecting OVCA at early stage and no imaging target(s) in the ovary corresponding to the elevated serum CA-125 levels has been defined. Our overall goal is to establish an early detection test for OVCA using vascular endothelial growth factor receptor -2 (VEGFR-2)- targeted ultrasound molecular imaging in association with serum anti-nuclear matrix protein (anti-NMP) antibodies (a marker of ovarian malignant transformation) and serum IL-16 levels (a marker of ovarian tumor-associated neoangiogenesis, TAN). This goal is being achieved by two specific aims. The results of specific Aim 1 suggest that VEGFR-2-targeted ultrasound molecular imaging enhances the detection of ovarian tumors in the laying hen model of spontaneous OVCA. Changes in OVCA related VEGFR-2-targeted ultrasound molecular imaging indices are associated with the prevalence of serum anti-NMP antibodies and the elevation of serum IL-16 levels.

15. SUBJECT TERMS
Ovarian cancer, Early detection, Ultrasound molecular imaging, VEGFR-2, animal model

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INTRODUCTION:

Ovarian cancer (OVCA) is the leading cause of death of women due to gynecological cancers [1]. While the survival rates of OVCA patients are remarkably high if the disease is detected at early stage (>80%), most cases of OVCA are diagnosed at late stages when the likelihood of successful therapy is very low[2]. Non-specificity of symptoms at early stage and the lack of an effective and specific early detection test make early detection of OVCA very difficult. Serum levels of CA-125 and traditional transvaginal ultrasound (TVUS) scanning are the currently available tests for the detection of OVCA. A combination of CA-125 and TVUS scanning failed to effectively detect OVCA at early stage as circulating levels of CA-125 are non-specific to early OVCA and traditional TVUS scanning cannot detect early OVCA related changes in the ovary[3]. ***Thus a fresh approach is needed***. Malignant nuclear transformation and tumor associated neoangiogenesis (TAN) are two of the earliest events in tumor initiation and development. During malignant transformation, the nuclei of cells undergo profound morphological changes in size and shape together with rearrangements in nuclear matrix proteins (NMPs). As a result, NMPs are shed into the circulation, in response to which the immune system produces anti-NMP antibodies. These NMPs and their corresponding anti-NMP antibodies are tissue specific[4]. Malignant nuclear transformation is followed by ovarian TAN. Development of angiogenic microvessels is the characteristic feature of ovarian TAN. These microvessels express vascular endothelial growth factor receptor-2 (VEGFR-2) which is an accepted marker of angiogenesis. Thus, serum anti-NMP antibodies and VEGFR-2 expressed by TAN vessels in the ovary represent potential markers for early ovarian tumor related changes, to be detected by serum analysis and by *in vivo* imaging, respectively, if an *in vivo* imaging probe can be developed. Although traditional Doppler ultrasound (DUS) scanning detects ovarian vascular structures, its limited resolution cannot detect early stage OVCA related ovarian TAN vessels. Our long term goal is to improve the detectability of ovarian TAN vessels at early stage OVCA by contrast enhanced VEGFR-2-targeted ultrasound molecular imaging. Due to the difficulty in identifying patients with early stage OVCA, in this project we are using laying hen model of spontaneous OVCA to achieve our goals [5, 6].

BODY: the research accomplishments associated with each task (task 2 for this report) outlined in the approved Statement of Work.

The overall hypothesis of this project is that *early stage OVCA lesions can be detected in laying hens using VEGFR-2 targeted contrast enhanced ultrasound molecular imaging in association with anti-NMP antibodies and serum levels of IL-16.* Part of this hypothesis including '*VEGFR-2-targeted ultrasound molecular imaging agents enhances signal intensity and detection of OVCA*' was examined in specific aim 1 described in Year-1 report submitted earlier. The remaining part of the hypothesis that '*VEGFR-2-targeted ultrasound molecular imaging agents can enhance detection of early stage OVCA*' is being tested in specific aim 2.

Task 2. VEGFR-2 targeted-ultrasound molecular imaging indices established in specific aim 1 will detect ovarian tumors in hens with circulatory anti-NMP antibodies. The sensitivity and specificity including the predictive values of VEGFR-2-targeted ultrasound molecular imaging and serum marker (IL-16 levels) diagnostic of OVCA are being examined.

2a. Selection and prospective monitoring of hens:

1. ***Selection of hens:*** Hens with low egg laying rates were examined for the presence of serum anti-NMPs (nuclear matrix proteins) by ELISA and ovarian abnormal morphology by VEGFR-2-targeted ultrasound molecular imaging indices established in Task 1. Hens with (n=50) and without (n=50)

circulating anti-NMP antibodies and without any ovarian abnormality (detectable by VEGFR-2 targeted ultrasound molecular imaging) were selected.

2. All selected hens including with or without serum anti-NMP antibodies were monitored and are being monitored prospectively.

2b. Prospective monitoring of hens by contrast enhanced VEGFR-2-targeted ultrasound molecular imaging for the detection of ovarian tumor or tumor associated neo-angiogenesis:

1. All hens were monitored and are being monitored prospectively at 15 weeks interval using the imaging indices established in Task 1.
2. Serum samples were collected at each scan to examine the prevalence of anti-NMP antibodies and the levels of circulatory IL-16.
3. Hens predicted to have early stage ovarian cancer by targeted imaging were sacrificed at the time of diagnosis and ovarian tissues were collected and processed for routine histology for microscopic examination for tumor lesions and their sub-types as well as for immunohistochemical and proteomic studies.
4. Ovarian sections were examined for the expression of molecular markers of TAN including VEGFR-2 and IL-16.
5. Immunohistochemical expression of VEGFR-2 expression was confirmed by immunoblotting.
6. To determine the period between the initiation of ovarian malignant lesions/microscopic carcinoma (as indicated by the prevalence of serum anti-NMP antibodies) to a solid tissue mass limited to a part of the ovary and detectable by VEGFR-2-targeted imaging, the length of prospective monitoring of hens have been extended to additional 3 months with the approval of the sponsor.
7. The predictive value of the VEGFR-2 target imaging indices diagnostic of early OVCA established in Task Aim 1 will be tested at the end of the Specific Aim 2.

Detailed Reports on the Accomplishments in Year-2 of the project life:

Specific Aim 2: VEGFR-2-targeted ultrasound molecular imaging indices and TAN (tumor associated neo-angiogenesis) indices established in specific aim 1 will detect ovarian tumors and ovarian TAN in anti-NMP antibody positive hens.

Animals:

3-4years old hens with low egg laying rates (<125 eggs/year) were selected from a flock of White Leghorn laying hens. Hens were further screened for the presence of serum anti-NMP antibodies and 50 hens with and 50 hens without anti-NMP antibodies were selected for prospective monitoring with VEGFR-2-targeted ultrasound molecular imaging for the detection of ovarian tumors using imaging indices established in Specific Aim 1. Hens were maintained and are being maintained under standard poultry husbandry practices and provided with food and water *ad libitum*.

Serum: Blood from all selected hens were collected at each scan, serum samples were separated and stored at -80^{o}C to determine the prevalence of anti-NMP antibodies and IL-16 levels by immunoassay later.

Contrast enhanced VEGFR-2-targeted ultrasound molecular imaging and image analysis:

Pre-targeted traditional ultrasound imaging: Pre-targeted traditional transvaginal ultrasound (TVUS) imaging was performed prior to the injection of VEGFR-2 targeted microbubbles using mechanical set up as reported earlier[7, 8]. Briefly, hens were humanely held by an assistant and imaged using an instrument equipped with a 5- to 7.5-MHz endovaginal transducer (MicroMaxx; SonoSite, Inc, Bothell, WA). Following immobilization of each hen, the transducer was inserted transvaginally and 2-dimensional (2D) transvaginal gray scale and pulsed Doppler sonographies were performed as reported earlier [7, 9]. Ovarian morphology was examined by gray scale sonography while ovarian vasculature was examined by Doppler ultrasound (DUS) imaging. DUS imaging indices including the resistive index (RI: [systolic velocity – diastolic velocity]/systolic velocity) and the pulsatility index (PI: [systolic velocity – diastolic velocity]/mean) were automatically calculated from at least two separate images from the same ovary as reported earlier [7, 9, 10]. The lower RI and PI values are used for analysis. All images were processed and digitally archived to review off-line later.

VEGFR-2-targeted imaging:
Contrast enhanced VEGFR-2-targeted ultrasound molecular imaging was performed immediately after pre-targeted imaging. Hens were injected with VEGFR-2 targeted microbubbles in a similar manner with identical mechanical settings as described above. The same pre-targeted area as well as relevant surrounding areas were imaged according to the instruction of the manufacturer of the targeted imaging agent and earlier report [8]. Within 5-7 min from the arrival, targeted microbubbles were accumulated at the target sites and unbound free microbubbles were washed out. All images were archived digitally in a still format as well as in real-time clips (15 minutes for each hen). The effects of targeted microbubbles were visually evaluated online during scanning and off-line afterward by reviewing the archived still images and video clips. The time of targeted imaging agent arrival (interval in seconds from administration of the contrast agent to its visual observation [in seconds]) in the ovaries with or without tumor was recorded in real time. After review of the complete clip, the region of interest (ROI) was selected. The average image intensity (in pixel values) over a ROI encompassing the tumor or a normal ovarian stroma was calculated using a computer assisted software (Microsuite^{TM} version Five, Olympus America, Inc., Canter Valley, PA) and diagnosed either to have early OVCA or normal based on the signal intensities established in specific aim 1. In addition, RI and PI values from post-targeted imaging were calculated.

Gross morphology and histopathology: Hens were euthanized following the diagnosis of ovarian tumors or ovarian TAN by contrast enhanced VEGFR-2-targeted ultrasound molecular imaging.

Following euthanasia, abnormal ovarian presentation including presence of ovarian solid mass with or without accompanied ascites as well as tumor metastasis (if any) to other organs was determined as reported earlier [6]. Tissues were processed for routine staining to determine histological sub-types of ovarian tumors as well as immunohistochemical and immunoblotting studies as reported previously [6].

Immunoassay and Immunoblotting: Serum prevalence of anti-NMP antibodies and IL-16 levels were determined by ELISA and confirmed by Western blotting as reported earlier [11, 12].

Immunohistochemistry: Paraffin and frozen sections of ovarian tumors were examined for the detection of VEGFR-2- and IL-16-expressing cells using anti-chicken VEGFR-2 and anti-IL-16 primary antibodies as reported earlier [11, 12]. The frequencies of VEGFR-2-expressing microvessels or IL-16 expressing cells were counted and analyzed as reported previously [11, 12]

Results: Changes in ovarian morphology relative to malignant transformation during the prospective monitoring detected by contrast enhanced VEGFR-2-targeted ultrasound molecular imaging are shown in **Figure 1**.

Detection of changes in ovarian morphology in hens with serum anti-NMP antibodies by prospective monitoring with VEGFR-2 targeted ultrasound imaging: All hens with circulating anti-NMP antibodies at initial scan had one or two developing large follicles without any detectable abnormalities in ovarian morphology. At 1st scan (15 weeks after the initial scan), similar to initial scan, all hens showed developing preovulatory follicles without any distinguishable abnormality including the presence of solid mass in the ovary. Serum IL-16 levels were lower and Doppler indices were higher than those diagnostic of ovarian cancer established in specific aim 1. At 2nd scan (30 weeks after the initial scan) although serum IL-16 levels increased in 15 hens with anti-NMP antibodies, no detectable changes in ovarian morphology was found during targeted scan. At 3rd scan (45 weeks from initial scan) additional 12 hens had increased serum IL-16 levels and all these hens (total 12+15 =27 of 50 hens including those with increased serum IL-16 levels during 2nd scan) had imaging intensities higher than that diagnostic of OVCA established in specific aim 1. Targeted imaging detected a small solid tissue mass limited to a part of their ovaries in 15 hens. In addition, 12 hens had multiple solid tissue masses accompanied with profuse ascites with imaging intensities higher than diagnostic levels established in specific aim 1. These tumor-like changes in the ovaries were associated with remarkable decrease in Doppler indices which were lower than those established in specific aim 1 for the diagnosis of early OVCA. All of these hens were euthanized. Furthermore, of 23 remaining hens, 6 hens showed increase in serum IL-16 levels at 3rd scan for the first time and these hens together with remaining 17 hens with normal IL-16 levels are being monitored to further confirm that serum IL-16 levels increase even before the tumor forms a solid tissue mass.

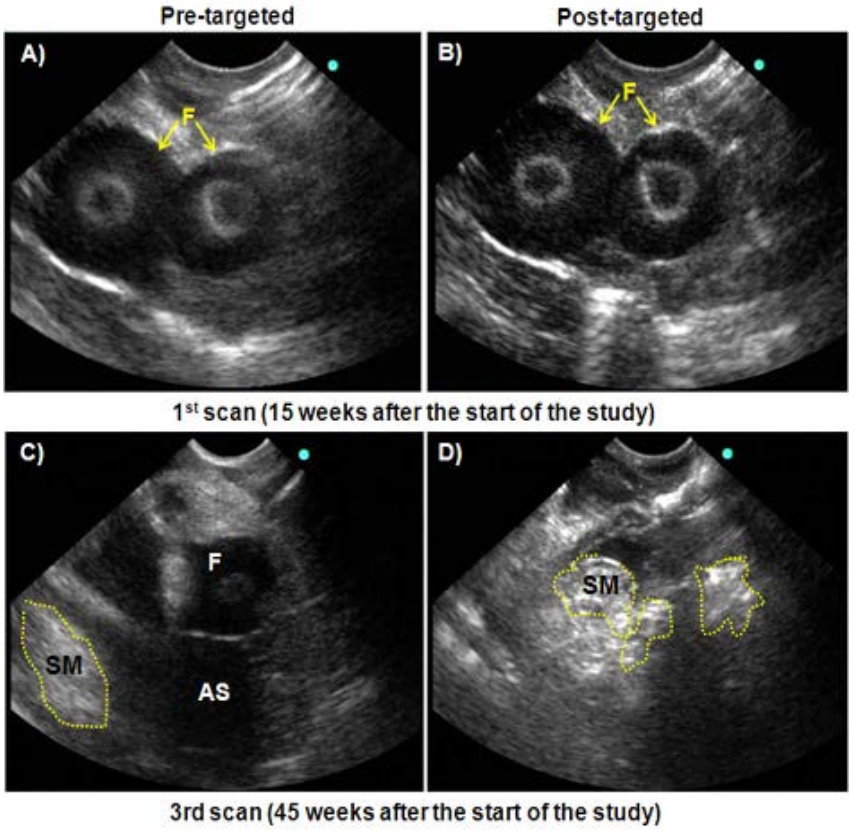

Figure 1. Prospective monitoring of hens to detect early changes associated with ovarian tumor development using VEGFR-2-targeted ultrasound molecular imaging agents. ***Top panel***: This hen was selected to have circulatory anti-NMP antibodies without any solid mass detectable by targeted ultrasound imaging. A-B) At 15 weeks after the start of the prospective monitoring, two developing preovulatory follicles (F) are seen in the ovary. The ovary appeared normal and compared with pre-targeted (A) remarkable increase in the signal intensity was not recorded during post-targeted imaging (B). ***Bottom panel***: The same ovary shown in top panel was scanned at 45 weeks after the start of the study. Pre-targeted sonogram (C) showed a suspected solid mass (SM) in a part the ovary with little to moderate ascites (AS) and a developing preovulatory follicle (F). Post-targeted scan (D) showed significant enhancement in signal intensity from areas of solid mass (yellow dotted circles).

Prospective imaging of hens without serum anti-NMP antibodies:
No significant changes were observed in ovarian morphology of hens without circulating anti-NMP antibodies from 1st scan to 3rd scan (**Figure 2**). Subsequent immunoassay showed neither the prevalence of anti-NMP

antibodies nor a significant increase in their serum IL-16 levels in "hens without anti-NMP antibody group" at 1st to 2nd scan (30 weeks from the start of the prospective study). At 3rd scan (45 weeks from the start of the study), anti-NMP antibodies were detected for the first time in 4 hens.

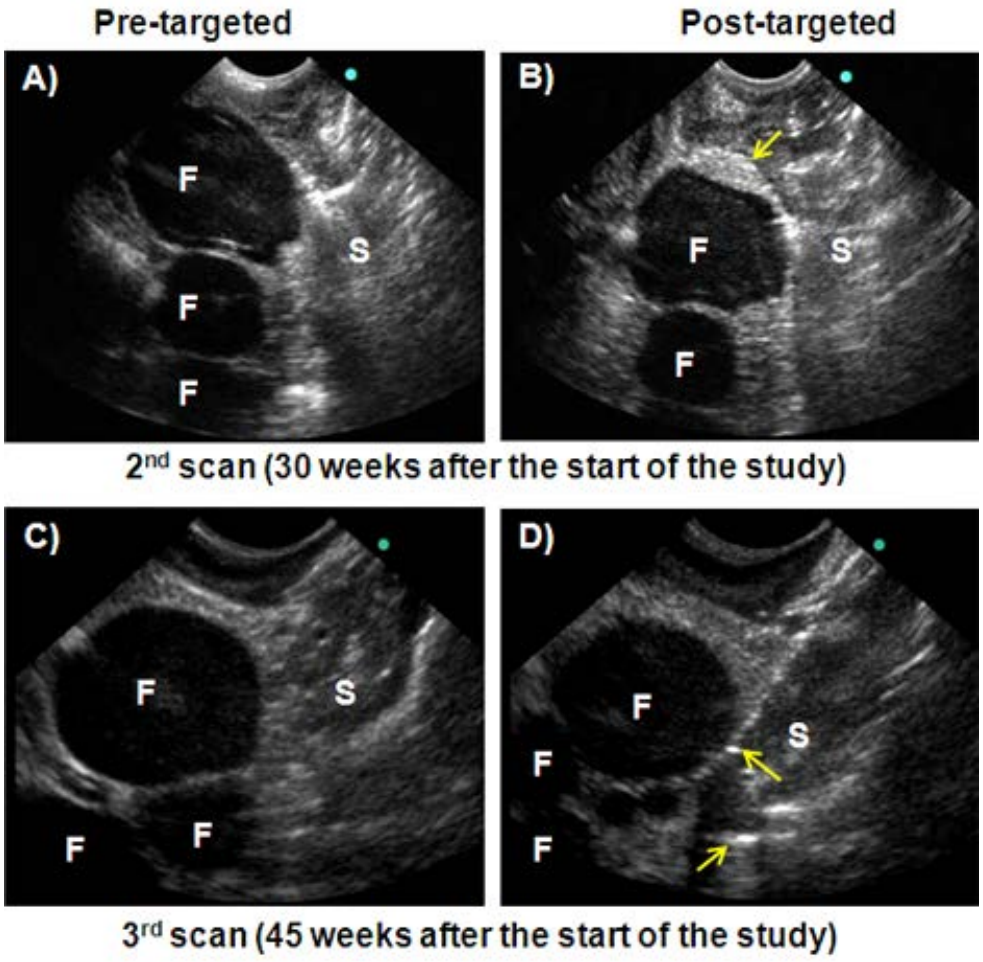

Figure 2. Prospective monitoring a hen without circulatory anti-NMP antibodies by VEGFR-2-targeted molecular imaging agents. ***Top panel***: A-B) At 30 weeks after the start of the prospective monitoring, sonograms revealed the ovary appeared normal containing 2-3 small and large developing follicles (*F*). Although intensity of signals increased alongside of the follicular wall (vascular areas) (arrow), compared with pre-targeted (A) remarkable increase in the signal intensity was not recorded during post-targeted imaging (B). ***Bottom panel***: The same ovary shown in top panel was scanned at 45 weeks after the start of the study. The ovary remained normal at 45 weeks and no abnormality was detected in targeted imaging. S= stroma. Arrows are the examples of potential blood vessels bound with the imaging agents.

Gross presentations and histopathological examinations of hens predicted to have ovarian tumors or ovarian TAN:

Following the diagnosis of suspected ovarian tumors by the VEGFR-2-targeted ultrasound molecular imaging, all hens were euthanized and gross morphology of ovaries were recorded and compared with the predictions of targeted imaging. Gross morphology including the presence of solid tumor mass in the ovary, extent of tumor metastasis and stages of OVCA as well as accompanying ascites was recorded. Ovaries with tumor and other relevant tissues including oviducts were harvested and processed for paraffin, frozen, proteomic and molecular biological studies. Histological sub-types of ovarian tumors were confirmed by routine histological examination with hematoxylin-eosin staining (**Figure 3**) as reported earlier [6]. As suggested by targeted ultrasound imaging, 15 hens had early stage OVCA (including 7 serous, 5 endometrioid, 3 mucinous) and tumors were limited to the ovary with no or very little ascites. In late stage OVCA (n = 12 hens including 4 serous, 4 endometrioid, 3 mucinous and 1 sero-mucinous mixed) was accompanied with moderate to profuse ascites and metastasized to peritoneal and abdominal organs.

Immunohistochemical expression of VEGFR-2:

Enhanced imaging intensity from VEGFR-2-targeted ultrasound molecular imaging as well as establishment of tumor associated neo-angiogenesis was confirmed by immunohistochemical localization of VEGFR-2. As

reported earlier [10], microvessels expressing VEGFR-2 were localized at the spaces between tumor glands (tumor vicinity, **Figure 4** ***top panel***). Malignant cells were also occasionally expressed VEGFR-2. The frequencies of VEGFR-2 expressing microvessels in hens with early stage OVCA were higher than those of diagnostic levels established in specific aim 1. The frequencies of VEGFR-2-expressing microvessels increased further as the tumor progressed to late stages. Differences in the frequencies of VEGFR-2 expressing microvessels were not observed among different histological sub-types of ovarian tumors. Increase in the population of ovarian VEGFR-2 expressing microvessels in association with tumor progression supported the increase in VEGFR-2-targeted imaging signal intensities in hens with late stage OVCA than hens with early stages.

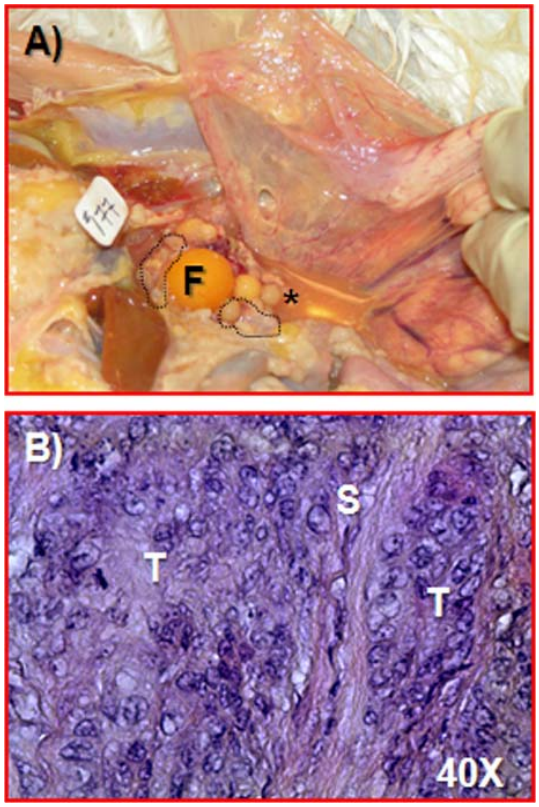

Figure 3. Gross and microscopic presentations of a hen ovary diagnosed to have ovarian tumor at early stage by VEGFR-2 targeted ultrasound molecular imaging shown in *figure 1*. A) As predicted during imaging, small solid masses (black dotted circles) were limited to parts of the ovary confirming early stage OVCA accompanied with little ascites (*****) and a developing preovulatory follicle (*F*). B) Routine staining confirmed the tumor (T) was of serous sub-type containing malignant cells with large pleomorphic nuclei surrounded by a sheath of fibromuscular layer in the stroma (S). 40X.

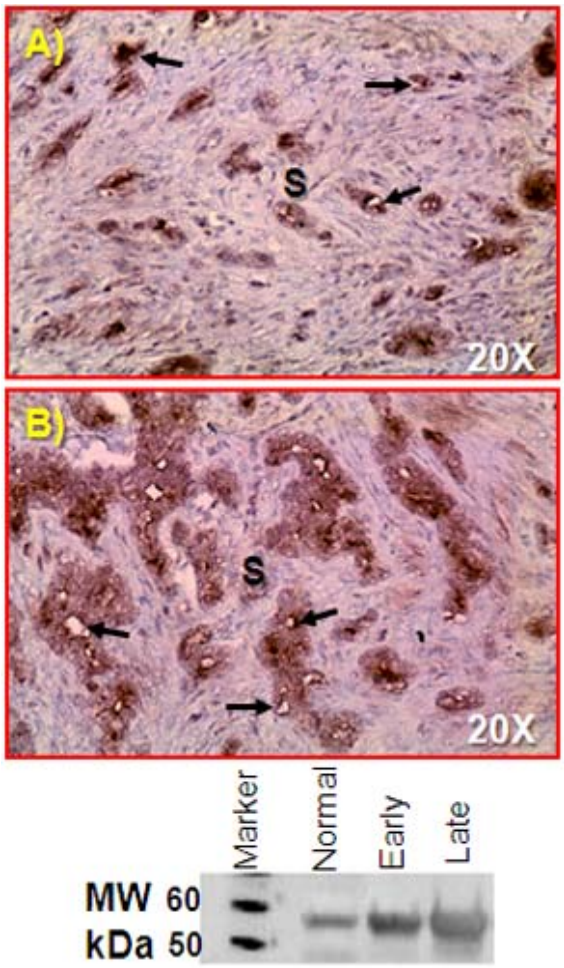

Figure 4. ***Top panel:*** A-B) Detection of VEGFR-2 expressing microvessels in ovarian tumors in hens. Immunopositive VEGFR-2 expressing microvessels were localized in the stroma (S) of ovaries with tumor at early stage (A) shown in figure 1 as well as at late stage of OVCA (B). Compared with early stage, more VEGFR-2 expressing microvessels are seen in late stage OVCA. 20X.
Bottom panel: Tissue expression of VEGFR-2 by normal and ovarian tumors at early and late stages. Western blotting showed increased expression of VEGFR-2 protein in association with OVCA development and progression. These results together with immunohistochemical detection support increased signal intensity due to binding of targeted agents with the increased number of VEGFR-2 expressing microvessels in tumors. 20X

Immunoblotting study detected VEGFR-2 protein of approx 55 kDa by ovarian tumors at early and late stages (**Figure 4, *bottom panel***). Compared with early stage, strong immunoreactive bands were observed for VEGFR-2 in ovarian tumors at late stage further confirming the increased expression as well as higher imaging signal intensities in hens with late stage OVCA than early stage OVCA.

Thus, VEGFR-2-targeted ultrasound molecular imaging enhances the detectability of ovarian tumors by transvaginal ultrasound imaging and represents a potential *in vivo* imaging probe for early detection of OVCA.

Detection of serum IL-16 levels and expression by ovarian tumors:
Serum levels of IL-16 were determined using Chicken IL-16 Vetset™ ELISA Kit (Kingfisher Biotech, St. Paul, MN) pre-coated with anti-Chicken IL-16 antibodies and chicken IL-16 as standards as per the manufacturer's instructions reported earlier and as reported previously [12]. The mean concentration of serum IL-16 in hens with early stage OVCA was higher than those of diagnostic levels established in specific aim 1 and increased further in hens at late stages of OVCA. However, the differences in serum IL-16 levels were not significant between the early and late stages OVCA as well as among the different histological subtypes of OVCA.

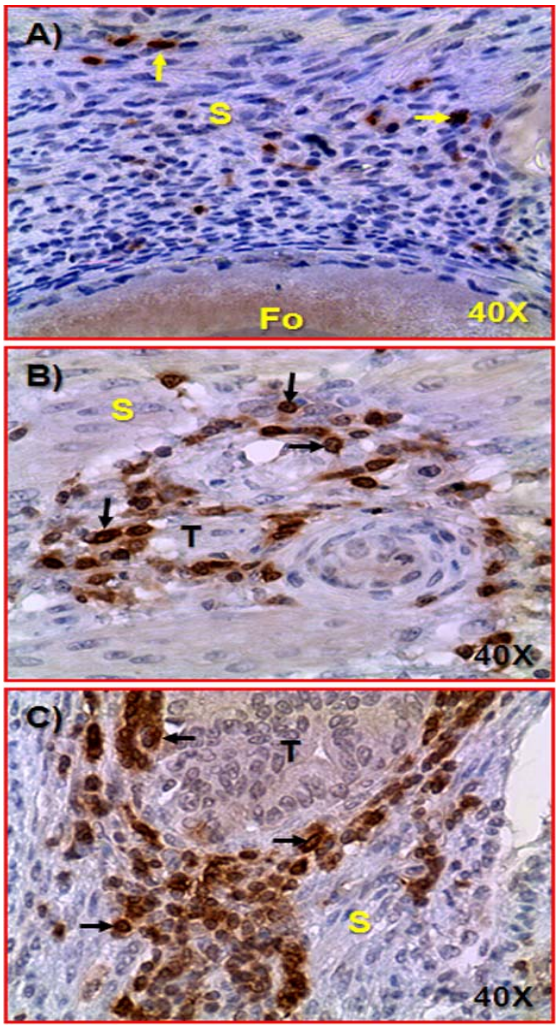

Figure 5. Changes in IL-16 expression in association with ovarian cancer (OVCA) development and progression. A) Section of a normal ovary showing very few immunostained IL-16-expressing cells in the stroma (S). B) section from an ovarian tumor at early stage of OVCA. Compared to normal, more IL-16-expressing cells seen in the stroma surrounding the tumor (T). C) section of an ovarian tumor at late stage. Many IL-16-expressing cells are seen in the stroma surrounding the tumor (T). Arrows indicate the examples of IL-16-expressing cells. 40X.

IL-16 was expressed by both the stromal cells including immune-cell like cells in the tumor stroma as well as occasionally by the ovarian malignant cells (**Figure 5**). A higher frequency of IL-16-expressing cells than those of diagnostic levels established in specific aim 1 was observed in hens with early stage OVCA and increased further in hens with late stage of OVCA.

These results suggest that increase in the frequency of IL-16 expressing cells in association with tumor initiation and progression may be a reason for the increase in serum IL-16 levels as the tumor progressed to later stages.

***Prevalence of serum anti-NMP antibodies in hens during prospective monitoring*:** All serum samples collected at different scan intervals were tested for the prevalence of anti-NMP antibodies using NMPs from archived normal or ovarian tumor NMPs collected from hens diagnosed for OVCA during prospective monitoring. The procedures for NMP collection and immunoassay were reported earlier [4, 13, 11]. Briefly, 96-well ELISA plates (NUNC) were coated with either tumor or normal ovarian NMPs and the immunoreactivities of serum samples from each hen collected at different scanning intervals were tested against the coated normal or ovarian tumor NMPs. Each serum sample was assayed in duplicate and the plates were read at 405nm in an ELISA plate reader (Softmax Pro, version 1.2.0, software; Molecular Devices, Sunnyvale, CA). Serum from young healthy hens with fully functional ovaries was used as negative control (established in earlier studies) for the presence of anti-NMP antibodies. Serum with optical density (OD) values higher than the control mean + 2SD (cut-off value) were considered positive for the presence of anti-NMP antibodies.

All hens in "*hens **with** anti-NMP antibodies*" group remained positive for anti-NMP antibodies at all scans so far completed (3rd scan at 45 weeks) including those diagnosed with OVCA. In contrast, neither the prevalence of

anti-NMP antibodies nor a significant increase in serum IL-16 levels was detected in "*hens **without** anti-NMP antibody group*" at 1st to 2nd scan (30 weeks from the start of the prospective study). At 3rd scan (45 weeks from the start of the study), anti-NMP antibodies were detected for the first time in 4 hens, however, neither serum IL-16 levels nor a tumor related changes in their ovarian morphology were detected by targeted ultrasound imaging. These hens and other remaining hens appeared normal are currently under monitoring.

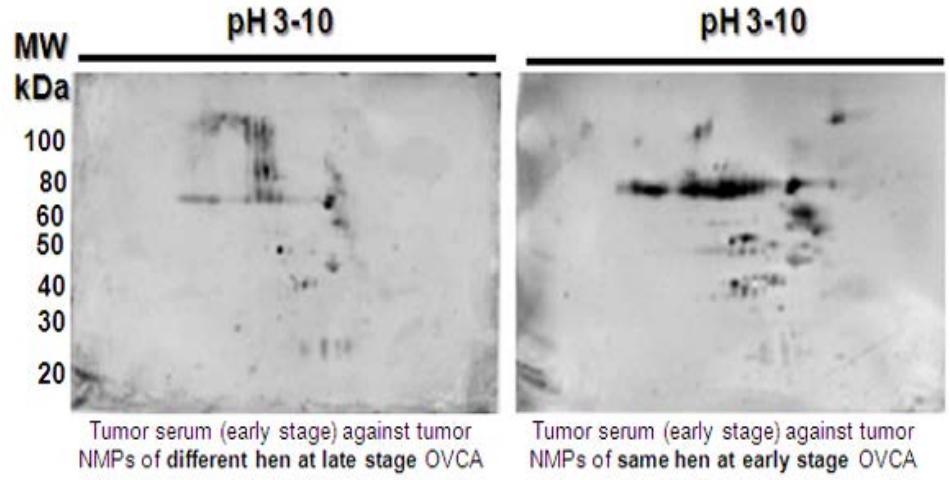

Figure 6. Immunoproteomic confirmation of prevalence of anti-NMP antibodies in sera of hens with ovarian malignant lesions. Tumor serum reacted specifically against tumor NMPs (of 30-100kDa sizes). Compared with heterogenous NMPs (left panel), serum reacted against more NMPs of homogenous tumor. These results confirmed the production of anti-NMP antibodies in response to a developing tumor.

Representative serum samples with positive reactivity against normal or tumor NMPs were analyzed by immunoproteomic study (2 dimensional-Western blotting, 2D-WB) to confirm immunoreactivities observed in ELISA.

Immunoreactive NMPs of different sizes (approx. 30-100kDa) were detected by 2D-WB (**Figure 6**) confirming the results of ELISA for the prevalence of anti-NMP antibodies in serum. Thus, these results suggest that NMPs are shed in serum during malignant transformation in the ovary and can be used to aid the early detection of ovarian cancer.

KEY RESEARCH ACCOMPLISHMENTS IN PROGRESS:

- Confirmation of the enhancement in ovarian tumor detection at early stage by VEGFR-2 targeted molecular imaging agents.
- Examination of the feasibility of serum anti-NMP antibodies as an aid to early detection of OVCA by VEGFR-2-targeted imaging are in progress.
- Testing the specificity of imaging signals due to the binding of targeted imaging agents with the microvessels in early stage ovarian tumors are in progress.
- Determination of time between the serum prevalence of anti-NMP antibodies as well as increased serum IL-16 levels and the formation of solid tumor in a part of the ovary (first detectable by targeted imaging) are in progress. This information will lead to the suitability of these markers for a screening protocol to detect early stage OVCA.

REPORTABLE OUTCOMES:

Presentation: Abstract published and presented:

1. Alongkronrusmee D, Bitterman P, Abramowicz JS, Bahr JM, Basu S, Grasso S, Sharma S, Rotmensch J and Barua. A (2013). "GRP78 in association with VEGFR-2 detects early stage ovarian cancer." In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr

6-10; Washington, DC. Philadelphia (PA): AACR; *Cancer Res* 2013; 73(8 Suppl): Abstract nr 4642. doi:10.1158/1538-7445.AM2013-4642 (appended in appendix 1)

Manuscript: One manuscript is under preparation and one is submitted for publication which under review.

CONCLUSIONS:

The results so far obtained with the progression of works under specific Aim 2 suggest that compared with the traditional ultrasound imaging, VEGFR-2-targeted ultrasound molecular imaging probes improved the detection of OVCA at early stage. This improvement in OVCA detectability was due to the enhanced ultrasound imaging signal intensity resulting from the bindings of imaging agents with their receptor VEGFR-2 expressed by the tumor microvessels. Furthermore, the results on anti-NMP antibodies confirmed that anti-NMP antibodies become prevalent before the tumor develops to an ultrasound-imaging-detectable mass in a part of the ovary. Similarly, serum levels of IL-16 also increased in association with malignant transformation in the ovary. With the completion of extended monitoring period these results will be further confirmed and the suitability of targeted imaging agents and serum markers for the early detection of OVCA will be determined.

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Poster Presentations - Biomarkers of Drug Response

Abstract 4642: GRP78 in association with VEGFR-2 detects early stage ovarian cancer.

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Background: The lack of an effective early detection test makes ovarian cancer (OVCA) a fatal malignancy of women. Changes in cellular metabolic processes during ovarian malignant transformation lead to the endoplasmic reticular (ER) and mitochondrial stress. Glucose-regulated protein of 78kDa (GRP78) is a marker of ER stress. Tumor-associated ER stress causes relocalization of GRP78 from ER to cell surface with an increase in its serum levels. Secreted GRP78 also stimulates tumor associated neoangiogenesis (TAN). Vascular endothelial growth factor receptor-2 (VEGFR-2) is a marker of ovarian TAN. Thus GRP78 represents a marker of ovarian malignant transformation and in association with VEGFR-2 may constitute an early detection test for OVCA. However, information on OVCA related changes in GRP78 expression and its association with ovarian TAN is unknown.

Objectives: The goals of this study were to examine (i) whether GRP78 expression increases during malignant changes in the ovary and (ii) whether the frequency of TAN vessels is associated with ovarian tumor-associated GRP78 expression at early stage of OVCA in the laying hen, a spontaneous model of human OVCA.

Materials and Methods: 3-4 years old White Leghorn laying hens with normal (n=25) or ovarian tumors (n=30) were selected by contrast enhanced transvaginal ultrasound scanning. Serum samples were collected, hens were euthanized, and ovarian tissues were processed for routine histology or immunohistochemistry (IHC) and protein extraction. Ovarian tumors were confirmed by gross morphology and microscopy. Expression of GRP78 by ovarian malignant cells and VEGFR-2 by TAN vessels was determined by IHC. IHC observations were confirmed by immunoproteomic studies.

Results: As compared with normal ovaries (n=25), the intensity of GRP78 expression was significantly ($p<0.001$) higher in hens with early stage OVCA (n=12) and increased further in hens with late stage OVCA (n=18) ($p<0.05$). GRP78 expression was higher in hens with serous OVCA followed by endometrioid OVCA and was least in hens with mucinous and clear cell OVCA irrespective of their stages of OVCA. A band of approximately 80kDa was observed for GRP78 in all ovaries examined and the patterns of expression were similar to that of IHC. The frequency of VEGFR-2 expressing TAN vessels was significantly ($p<0.001$) higher in OVCA hens than in normal hens. Increase in the frequency of VEGFR-2 expressing TAN vessels was positively correlated with the intensity of GRP78 expression.

Conclusion: The results of this study suggest that increase in the GRP78 expression is associated with ovarian malignant transformation, and this enhanced expression may stimulate ovarian tumor-associated neoangiogenesis. Thus GRP78 in combination with VEGFR-2 may constitute an early detection test for OVCA. These results will constitute a foundation for a clinical study to establish an early detection test for OVCA. Support: DOD Award # W81XWH-11-1-0510.

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